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RESEARCH ARTICLE

CKS Proteins Promote Checkpoint Recovery by Stimulating Phosphorylation of Treslin

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ABSTRACT CKS proteins are small (9-kDa) polypeptides that bind to a subset of the cyclin-dependent kinases. The two paralogs expressed in mammals, Cks1 and Cks2, share an overlapping function that is essential for early development. However, both proteins are frequently overexpressed in human malignancy. It has been shown that CKS protein overexpression overrides the replication stress checkpoint, promoting continued origin firing. This finding has led to the proposal that CKS protein-dependent checkpoint override allows premalignant cells to evade oncogene stress barriers, providing a causal link to oncogenesis. Here, we provide mechanistic insight into how overexpression of CKS proteins promotes override of the replication stress checkpoint. We show that CKS proteins greatly enhance the ability of Cdk2 to phosphorylate the key replication initiation protein treslin *in vitro*. Furthermore, stimulation of treslin phosphorylation does not occur by the canonical adapter mechanism demonstrated for other substrates, as cyclin-dependent kinase (CDK) binding-defective mutants are capable of stimulating treslin phosphorylation. This effect is recapitulated *in vivo*, where silencing of Cks1 and Cks2 decreases treslin phosphorylation, and overexpression of wild-type or CDK binding-defective Cks2 prevents checkpoint-dependent dephosphorylation of treslin. Finally, we provide evidence that the role of CKS protein-dependent checkpoint override involves recovery from checkpoint-mediated arrest of DNA replication.

KEYWORDS CKS protein, treslin, treslin phosphorylation, replication stress checkpoint, checkpoint recovery

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CKS proteins were discovered in budding yeast and fission yeast based on genetic interactions with *CDK1* mutants (*cdc28* in budding yeast and *cdc2* in fission yeast) (1, 2). Based on homology, similar proteins were identified in a variety of eukaryotes, including two paralogs in vertebrates, Cks1 and Cks2 (3). Although these small conserved proteins were shown, shortly after their discovery, to bind to a subset of cyclin-dependent kinases (CDKs) (4), their function(s) remained obscure until recently. The crystallographically determined geometry of Cks1 bound to Cdk2 suggested an adaptor function, promoting targeting of the kinase to substrates (5). The fact that CKS proteins possess a phosphate binding site has led to the hypothesis that CKS proteins can tether CDKs to substrates already primed by phosphorylation for efficient subsequent multiphosphorylation. Indeed, this mechanism has been borne out for some substrates, such as the yeast CDK inhibitors Sic1 and Far1 (6, 7), and is probably the case for a variety of other CDK substrates in vertebrates, such as Cdc27, Cdc25, Wee1, and Myt1 (8, 9). However, a number of other well-characterized adapter functions have been attributed to CKS proteins, all presumably stemming from their ability to form relatively high-affinity complexes with CDKs. CKS proteins are critical for efficient docking of Cdk2-cyclin A complexes to the ubiquitin ligase anaphase-promoting

complex/cyclosome (APC/C) for the metaphase ubiquitin-dependent proteolysis of cyclin A (10). The paralog Cks1 is essential for the binding of the SCF ubiquitin ligase specificity factor Skp2 to the CDK-bound inhibitor $p27^{Kip1}$ for ubiquitylation and degradation (11, 12). Perhaps the most unexpected roles for CKS proteins are in the realm of transcriptional regulation. In budding yeast, it was found that the Cks1-Cdk1 complex has a kinase-independent role in nucleosome eviction during rapid transcriptional induction (13, 14). This function requires the Paf1 elongation complex and the 19S proteasome particle (15). In mammalian cells, the essential redundant function of Cks1 and Cks2 is the expression of mRNAs encoding Cdk1, cyclin B1, and cyclin A2 (16). Although the molecular mechanism remains to be elucidated, it may involve a chromatin remodeling function analogous to what has been observed in yeast.

In addition to their normal cellular roles, CKS proteins are likely to have roles in oncogenesis. Cks1 and/or Cks2 are frequently overexpressed in a broad spectrum of malignancies (17–30). It has been hypothesized that Cks1 overexpression is linked to its role in degradation of the CDK inhibitor $p27^{Kip1}$, as low p27 levels have been associated with aggressive malignancy in various types of cancer. However, attempts to correlate high Cks1 levels with low p27 levels in tumors have given mixed results (31–33).

Another potential insight into the role of Cks proteins in oncogenesis comes from the observation that Cks1 or Cks2, when overexpressed, overrides the replication stress checkpoint (34). Whereas triggering of the replication stress checkpoint normally prevents subsequent replication origin firing, cells overexpressing either Cks1 or Cks2 continue to fire origins, even though checkpoint signaling is intact. This observation led us to speculate that CKS protein overexpression allows premalignant cells to evade DNA damage checkpoint barriers triggered as the first line of defense in response to activated or overexpressed oncoproteins. Indeed, we observed that there was a strong correlation in breast tumors between overexpression of cyclin E (34), an oncoprotein that causes replication stress, and either Cks1 or Cks2. The replication stress checkpoint detects single-stranded DNA resulting from stalled or collapsed replication forks and signals to Cdk2 by promoting the degradation of the CDK phosphatase CDC25A (35–37). As a result, Cdk2 accumulates in its tyrosine 15-phosphorylated inactive form (38). The link between Cdk2 activity and origin firing is likely to be the protein treslin (39, 40). The assembly of an active replicative helicase requires the CDK-dependent phosphorylation of treslin on serine 1000 (S1000). Phosphorylated treslin then binds to BRCT domains I and II of the protein TopBP1, leading to the recruitment of the initiator protein Cdc45. This is a highly conserved pathway, where in yeast Sld3 the treslin ortholog binds to Dbp11, the TopBP1 ortholog, in a CDK-dependent manner to initiate replication (41, 42). Checkpoint-mediated inhibition of Cdk2 presumably leads to reversal of these events, precluding subsequent origin firing.

Since CKS protein overexpression permits origin firing in the context of an active replication stress checkpoint, we sought to determine if there is a link between CKS protein function and treslin phosphorylation. We found that both Cks1 and Cks2 greatly enhance the efficiency of treslin phosphorylation by Cdk2 *in vitro* in a reconstituted reaction using purified proteins as well as in cultured cells. Interestingly, the stimulation of treslin phosphorylation does not occur by a mechanism whereby the CKS protein serves as a canonical CDK substrate adapter, as previously reported, but rather by improving the efficiency of CDK-treslin interaction either by remodeling treslin or by serving as a noncanonical Cdk2 adapter. Furthermore, we provide evidence that the function of CKS-enhanced phosphorylation of treslin entails recovery from the replication stress checkpoint.

RESULTS

Both Cks1 and Cks2 stimulate phosphorylation of treslin by Cdk2. In order to determine whether Cks1 and/or Cks2 has a role in treslin phosphorylation on serine 1000 (S1000), we developed an *in vitro* system using purified proteins. As a surrogate substrate for treslin, we used a glutathione *S*-transferase (GST) fusion to a 23-amino-acid (aa) treslin peptide centered around S1000 [aa 989 to 1011; termed treslin

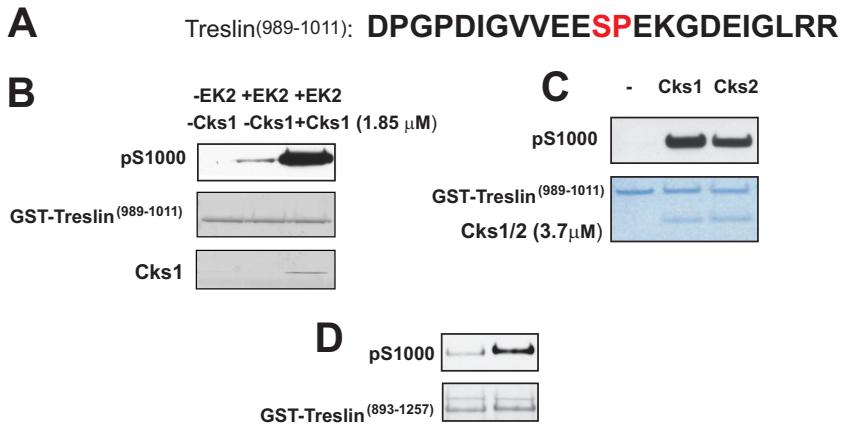

FIG 1 Cks1 and Cks2 stimulate treslin phosphorylation *in vitro*. (A) GST-treslin substrate. A 23-amino-acid peptide centered around treslin S1000 fused to GST was used as a Cdk2 substrate for most *in vitro* phosphorylation experiments described. (B) Stimulation of GST-treslin phosphorylation by Cks1. *In vitro* kinase reactions were carried out using a 23-amino-acid peptide flanking the S1000 phosphorylation site of treslin fused to GST. Each 30-μl reaction mixture contained 17 nM recombinant cyclin E/Cdk2, 1.1 μM GST-treslin, and the indicated concentration of Cks1. Phosphorylation of the treslin substrate was detected after SDS-PAGE and Western blotting using an antibody that recognizes phosphorylated S1000 of treslin. Cks1 and GST-treslin were detected by amido black staining. (C) Cks1 and Cks2 both stimulate phosphorylation of GST-treslin. Reaction mixtures like those described for panel B were set up with 3.7 μM Cks1 or Cks2. (D) Cks1 stimulates Cdk2-mediated phosphorylation of a larger fragment of treslin fused to GFP (aa 893 to 1257). Reaction mixtures were like those described for panel B but with 0.185 μM Cks1.

(989-1011)] (40) (Fig. 1A). We purified active cyclin E-Cdk2 from an *Escherichia coli* strain that also expresses the yeast Cak1 kinase, which phosphorylates the CDK T-loop-producing active enzyme (43). Cks1 and Cks2 were also purified from *E. coli*. Phosphorylation of the treslin substrate after incubation with Cdk2 was detected using a phosphoepitope-specific antibody (40). GST-treslin and Cks1/2 were detected by amido black staining of blots. Cyclin E-Cdk2 was capable of phosphorylation of the treslin peptide without any additional proteins. However, addition of Cks1 or Cks2 stimulated treslin phosphorylation by 10- to 20-fold (Fig. 1B and C). Cks1 also stimulated phosphorylation of a larger treslin polypeptide fused to GST (aa 893 to 1257) (Fig. 1D) (40). In order to rule out that the stimulation of treslin peptide phosphorylation was due to an interaction between CKS protein and the GST tag, we carried out a parallel experiment using a pure synthetic peptide [treslin (997-1008)] and determined the degree of phosphorylation by mass spectroscopy (MS). Cks1 increased phosphorylation of this peptide by Cdk2 by 10-fold (Fig. 2), similar to what was observed using GST fusion and Western blotting. Therefore, CKS proteins stimulate treslin peptide phosphorylation by interacting directly with treslin sequences.

Dose-dependent phosphorylation of treslin peptide. A kinase assay, as described above, was performed with Cks1 concentrations ranging from 1.85 nM to 3.7 μM (Fig. 3A). The reaction mixture concentrations of Cdk2 and GST-treslin peptide were 17 nM and 1.1 μM, respectively. As can be seen in Fig. 3A, the phosphorylation of treslin peptide saturates at stoichiometry of Cks1 approximately equivalent to that of GST-treslin peptide and well in excess of that of Cdk2. On the other hand, the 50% effective concentration (EC_{50}) of Cks1-mediated stimulation of treslin phosphorylation is 90 nM (Fig. 3B), similar to the previously determined dissociation constant (K_d) of Cks1 binding to Cdk2 (77 nM) (5). Therefore, these data are consistent with Cks1 functioning through binding to Cdk2 or as a stoichiometric substrate adapter. In order to investigate this finding further, we determined the smallest treslin peptide capable of CKS protein-stimulated phosphorylation. An 8-amino-acid peptide (aa 997 to 1004) fused to GST exhibited CKS-enhanced phosphorylation similar to that of treslin (989-1011) (Fig. 3C). Considering that Cks1 has been shown to bind to the C-lobe of Cdk2 distal from the

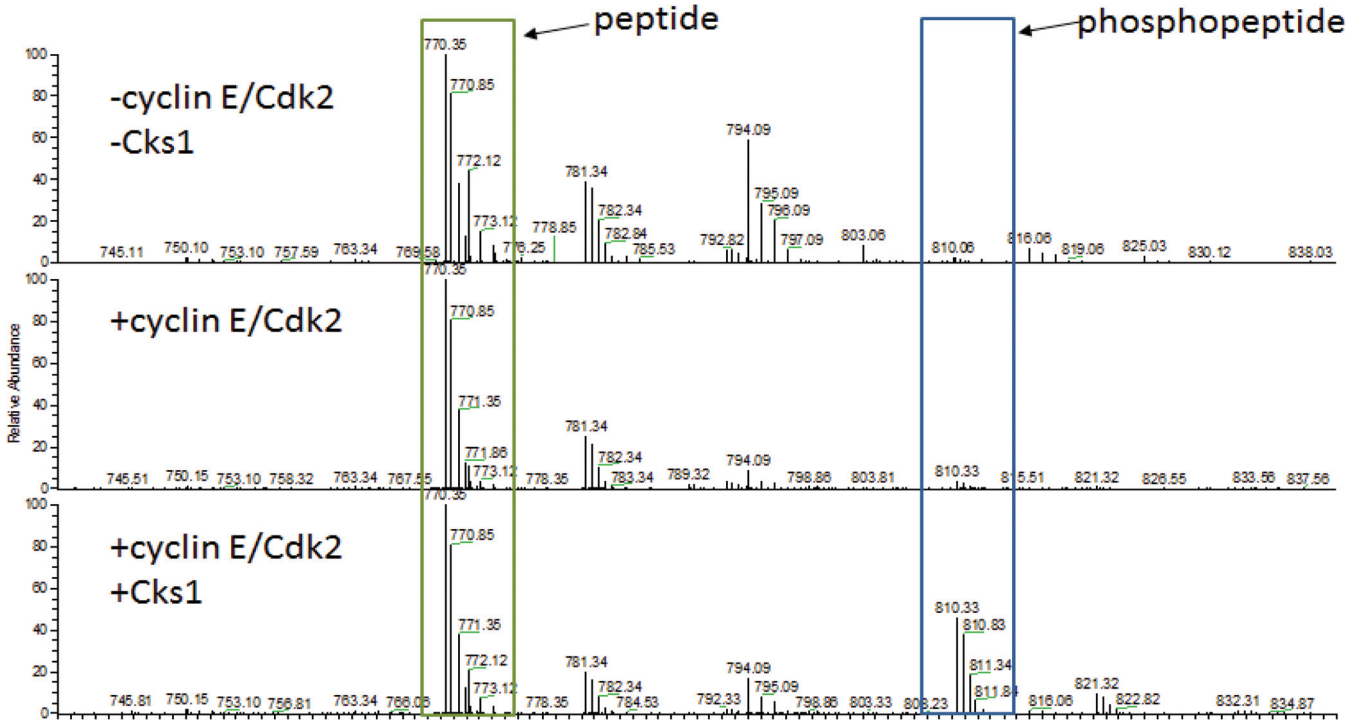

FIG 2 Cks1 enhances phosphorylation of a small synthetic treslin peptide. Kinase reactions were as described in the legend to Fig. 1. A synthetic peptide corresponding to treslin amino acids 997 to 1008 was incubated with buffer only, with cyclin E-Cdk2 alone, or with cyclin E-Cdk2 and Cks1 (1.1 μM). The three mixtures were infused into an Orbitrap mass spectrometer using the ESI method, and both MS1 and MS2 were recorded. MS peaks representing unmodified peptide and phosphopeptide are indicated.

active site (5), it would be impossible for an 8-amino-acid peptide with an internal phosphorylation site to bind simultaneously to the active site and to CDK-bound Cks1. We then carried out an experiment to determine whether Cks1 or Cks2 could form a complex directly with treslin in the absence of Cdk2. Thermal shift analysis was carried out on CKS protein-treslin peptide mixtures. Incubation with synthetic treslin peptide

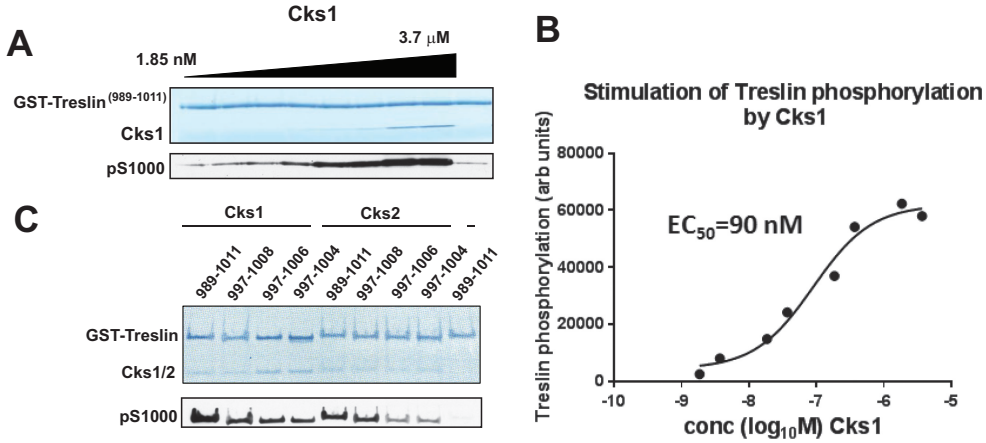

FIG 3 CKS proteins stimulate treslin phosphorylation by a noncanonical mechanism. (A) Dose-dependent Cks1 stimulation of GST-treslin (989-1011) phosphorylation. The kinase assay was performed as described in the legend to Fig. 1. Cks1 concentrations ranged from 1.85 nM (left) to 3.7 μM (second from the right). The area on the far right has no Cks1. (B) Quantification of data shown in panel A using Image J and GraphPad Prism software. arb, arbitrary; conc, concentration. (C) CKS proteins stimulate phosphorylation of 8-amino-acid peptides flanking the treslin S1000 phosphorylation site. GST fusions corresponding to 23-, 12-, 10-, and 8-amino-acid treslin peptides were incubated with Cks1 or Cks2, as indicated, and cyclin E/Cdk2 as described in the legend to Fig. 1. The lane on the far right corresponds to a reaction without CKS protein.

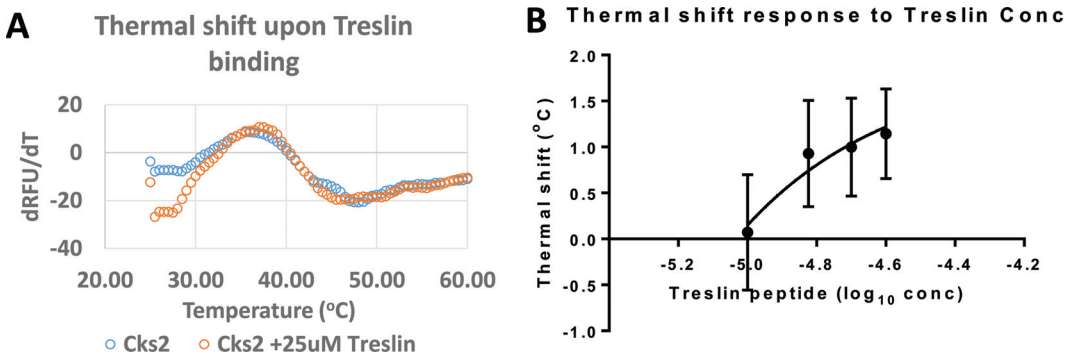

FIG 4 Cks2 binds weakly to treslin in the absence of Cdk2. (A) Derivatives of melting curves of Cks2 alone and Cks2 with 25 μM treslin (997-1008) peptide in kinase buffer. The peak of the derivative curve corresponds to the T_m. (B) Determination of the K_d of treslin peptide for Cks2 binding by alteration of the Cks2 melting curve. T_m values were determined using the indicated concentrations of treslin (997-1008) peptide. Treslin (997-1008) alone produces no signal in this assay. Average values ($n = 8$) were plotted and analyzed using GraphPad Prism. Error bars correspond to SD.

in buffer used for kinase reactions reproducibly altered the thermal melting curve of Cks2 by 1.14°C ($P = 0.002$ by single-tailed t test) (Fig. 4A), indicating direct interaction. A small thermal shift that did not achieve significance was observed for Cks1 under the same conditions at the maximum treslin peptide concentration permissible for the assay (25 μM). However, a thermal shift was observed using different buffer conditions (data not shown due to lack of relevance to the kinase assay). The thermal shift assay was then used to determine an approximate K_d (16 μM) for treslin peptide binding to Cks2 (Fig. 4B). This weak interaction is not consistent with the EC_{50} determined for CKS-dependent stimulation of treslin phosphorylation (90 nM) and suggests a mechanism more complex than simple binding to and remodeling of the phosphorylation site on treslin (see Discussion).

Neither canonical CDK binding nor phosphate binding is required for stimulation of treslin phosphorylation. In order to confirm that CDK binding is not important for stimulation of treslin phosphorylation by Cdk2, we compared stimulation by wild-type Cks2 and a mutant (Cks2 E63Q) previously shown to be defective in CDK binding (Fig. 5A). There was no defect in stimulation of treslin phosphorylation at high concentrations of Cks2 E63Q and a slight defect at low concentrations. To confirm that the E63Q mutation prevents binding to CKS proteins, we carried out a pulldown experiment using Flag-Cks1 and Flag-Cks1 E63Q with anti-Flag beads. Cyclin E-Cdk2 was retained on the beads only if mixed with wild-type Cks1 but not with Cks1 E63Q (Fig. 5B). Although not apparent in Fig. 5B, it has been shown that the mutant paralog Cks2 E63Q has a residual, extremely weak affinity for Cdk2 based on surface plasmon resonance measurements (44). Therefore, we combined the E63Q mutation with another mutation shown to completely eliminate CDK binding (45), creating the double mutant Cks1 E63Q H60A. Figure 5C compares wild-type Cks1, Cks1 E63Q, Cks1 H60A, and Cks1 E63Q H60A at 1.85 μM for ability to stimulate treslin phosphorylation. As is evident, all mutants are indistinguishable from the wild type, indicating that canonical Cdk2 binding is not required for stimulation of treslin phosphorylation. To rule out that stimulation of treslin phosphorylation is simply a function of protein concentration in the reaction mixture, 10 μg/ml bovine serum albumin (equivalent to 1.1 μM CKS protein) was substituted for CKS protein (Fig. 5A). No stimulation of treslin phosphorylation was observed.

Since the GST-treslin peptide contains only one CDK phosphorylation site, it was unlikely that a phosphate binding mutant would affect phosphorylation unless these residues are directly involved in treslin binding by another mechanism. Consistent with this expectation, a triple mutant (K11E, S51E, R71A) shown to abolish phosphate binding (5) was not defective in stimulation of GST-treslin peptide phosphorylation (Fig. 5D).

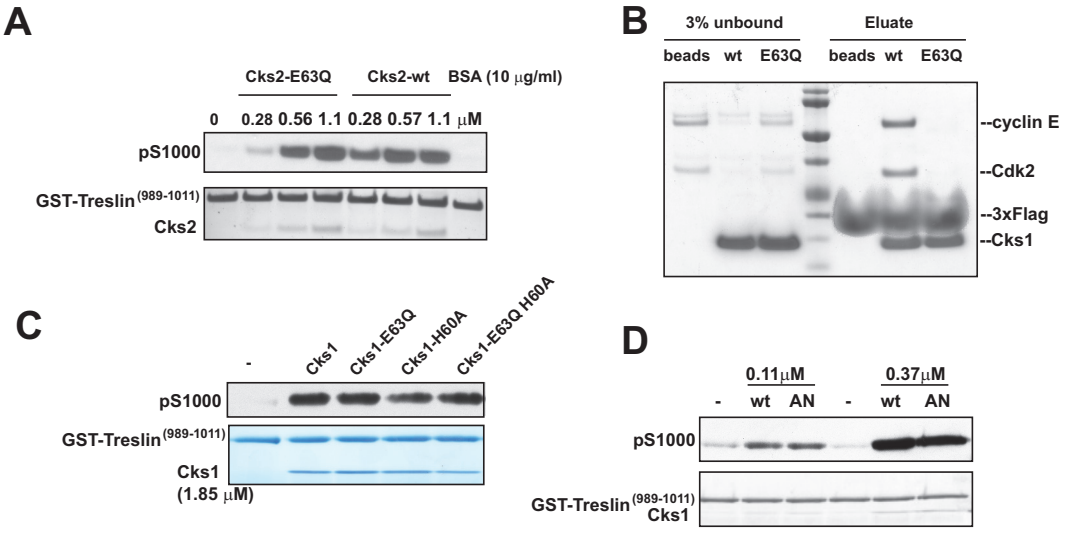

FIG 5 CKS mutants defective in CDK binding and phosphate binding can still stimulate treslin phosphorylation. (A) Cks2 E63Q, shown to be defective in CDK binding, is not defective in stimulating GST-treslin (989-1011) phosphorylation. Reactions and analysis were carried out as described in the legend to Fig. 1. Concentrations of wild-type and mutant Cks2 are indicated. The last lane contains 300 ng (10 μg/ml) of bovine serum albumin, confirming the lack of a nonspecific carrier protein effect. wt, wild type. (B) Cks1 E63Q is defective in binding to Cdk2. Cyclin E-Cdk2 was incubated with anti-Flag epitope (M2) beads alone or in the presence wild-type or mutant (E63Q) Flag-tagged Cks1. Beads were then washed and incubated with 3× Flag peptide to elute bound Flag-Cks1 and associated proteins. Eluates were analyzed by SDS-PAGE and proteins revealed by Coomassie blue staining. Lanes on the left correspond to supernatant after removal of beads. (C) A CDK binding null mutant of Cks1 containing 2 individual point mutations is not defective in stimulating treslin phosphorylation. The assay was carried out as described in the legend to Fig. 1. (D) A Cks1 mutant defective in anion binding (AN) is competent to stimulate treslin phosphorylation. A triple mutant defective in anion (phosphate) binding (K11E, S51E, R71A) was compared to wild-type Cks1 using the assay described in the legend to Fig. 1.

Cks1 and Cks2 alter the K_m of the treslin phosphorylation reaction. If CKS protein functions to improve the ability of treslin to interact productively with Cdk2, then it should reduce the K_m of the reaction. We therefore determined the K_m for treslin in the absence of CKS protein versus that in the presence of saturating concentrations of Cks1 or Cks2. For this experiment, mutant protein defective in CDK binding (E63Q) was used to ensure that effects observed would be related to interactions with treslin rather than Cdk2. Without CKS protein in the reaction, we could not observe saturation at concentrations that were permissible in the reaction (Fig. 6). However, in the presence of Cks1 or Cks2, saturation was approached and K_ms could be determined by curve fitting (59 μM for Cks1 and 16 μM for Cks2) (Fig. 6). The lower K_m in the presence of Cks2 compared to Cks1 is consistent with the apparent lower affinity of Cks1 for treslin based on the thermal shift experiments. These data suggest that CKS protein binding improves the efficiency of treslin association with Cdk2, although a simple

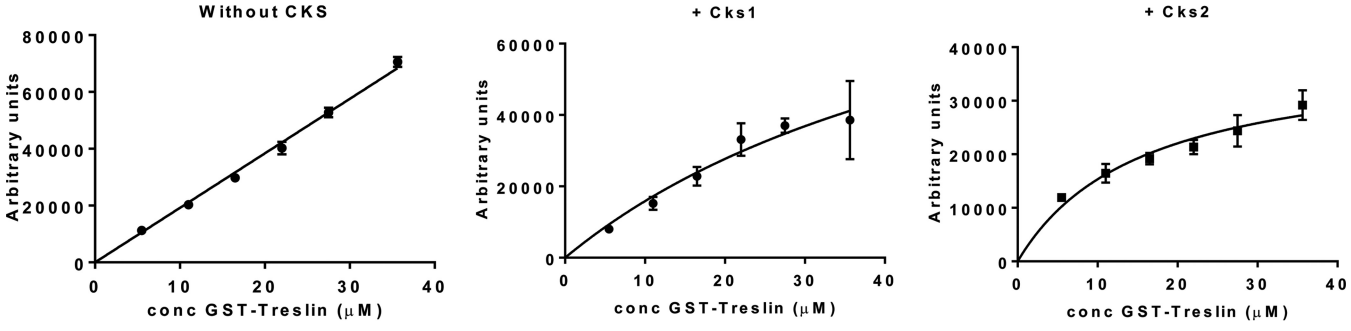

FIG 6 Determination of the K_m for treslin phosphorylation in the presence and absence of CKS proteins. Saturation curves for cyclin E-Cdk2 phosphorylation of GST-treslin in the absence of CKS proteins or in the presence of 25 μM Cks1 or Cks2 is shown. The K_m was determined by curve fitting using GraphPad Prism software. Error bars correspond to SD.

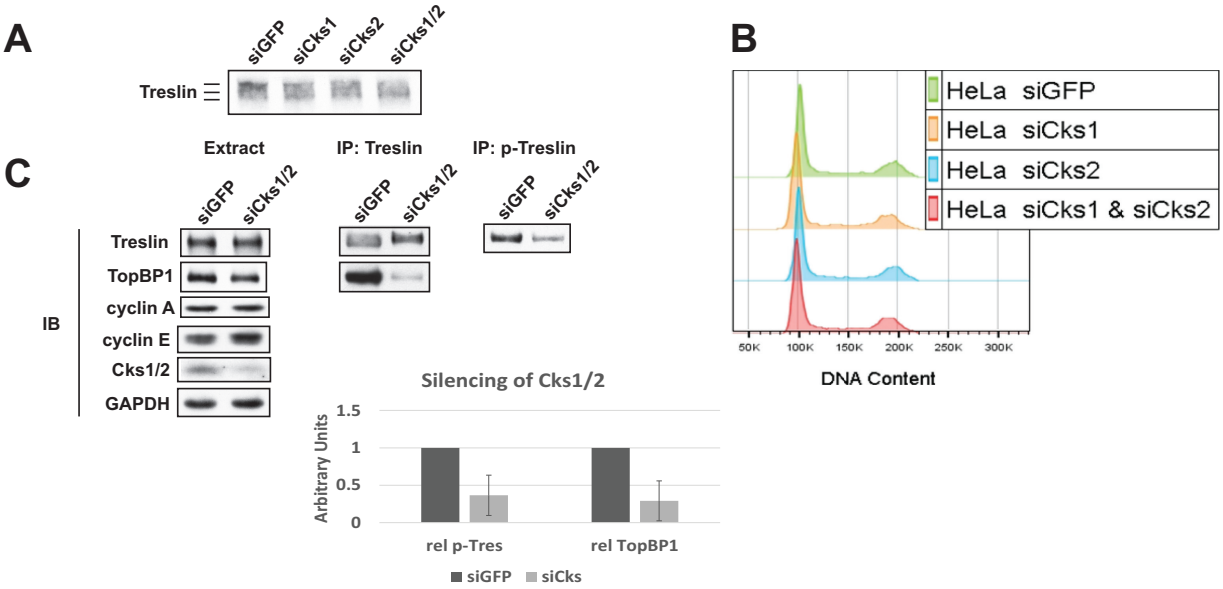

FIG 7 Silencing of CKS proteins leads to reduced phosphorylation of treslin in cells. (A) siRNA-mediated silencing of Cks1, Cks2, or both leads to increased mobility of treslin in SDS-PAGE. HeLa cells were transfected with siRNAs corresponding to GFP (control), Cks1, Cks2, or a mixture of Cks1 and Cks2 siRNAs. Cells were harvested after 24 h and extracts analyzed by long runs on 3 to 8% polyacrylamide gels to resolve differentially phosphorylated species of treslin. Treslin was detected after blotting using antitreslin antibody. Arrows indicate the three most predominant treslin species. (B) Flow-cytometric analysis of HeLa cells 24 h after siRNA transfection. (C) siRNA-mediated silencing of Cks1 and Cks2 leads to a reduction in treslin S1000 phosphorylation and TopBP1 binding to treslin. HeLa cells were transfected with siRNAs corresponding to GFP (control) and both Cks1 and Cks2. Twenty-four hours later, extracts were prepared and immunoprecipitated (IP) with either treslin antibody or treslin phospho-S1000 antibody. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting as indicated. TopBP1 and treslin were analyzed on the same blot by serial incubation with TopBP1 and treslin antibodies. The bar graph corresponds to quantification of three independent biological experiments. "rel p-Tres" and "rel TopBP1" refer to the ratios of phosphotreslin and TopBP1 to total treslin, respectively. Error bars represent SD. (Left) Extracts were also analyzed for the indicated proteins by immunoblotting. Glyceraldehyde phosphate dehydrogenase (GAPDH) represents the loading control.

model of CKS protein binding to treslin prior to interaction with Cdk2 is inconsistent with the determined K_d (see Discussion).

CKS protein function is important for treslin phosphorylation in cells. To determine whether CKS proteins have a role in treslin phosphorylation in cells, as suggested by the biochemical studies described above, we silenced Cks1, Cks2, or both Cks1 and Cks2 simultaneously in HeLa cells using RNA interference (RNAi) technology. Extracts were separated by SDS-PAGE under conditions designed to resolve high-molecular-mass proteins such as treslin (apparent molecular mass of ~230 kDa). Upon immunoblotting using these conditions, treslin resolves into several species corresponding to different phosphorylation states (46). In most cases, phosphorylation confers lower mobility to proteins. Accordingly, silencing of Cks1 or Cks2 modestly shifted the array of treslin species to higher mobility (Fig. 7A). However, simultaneous silencing of both Cks1 and Cks2 produced a much more significant mobility shift (Fig. 7A). These data suggest that Cks1 and Cks2 contribute to treslin phosphorylation in cycling cells. However, treslin is a large protein with numerous CDK consensus potential phosphorylation sites, and a shift in mobility does not directly indicate the phosphorylation status of S1000, the key site for origin firing (40). We therefore carried out an experiment to directly assess the phosphorylation status of treslin S1000. Extracts prepared from control HeLa cells and HeLa cells silenced for Cks1 and Cks2 were immunoprecipitated with antitreslin and anti-phospho-S1000 antibodies, respectively, and precipitates were subjected to SDS-PAGE and immunoblotting using antitreslin antibody. Comparing the ratio of treslin p-S1000 to total treslin immunoprecipitated indicated that silencing of Cks1 and Cks2 led to an ~70% reduction in phosphorylation of treslin on S1000 ($P = 0.0076$ by single-tailed t test) (Fig. 7C). Binding of TopBP1 can also serve as an indicator for treslin S1000 phosphorylation (40). We therefore analyzed the amount of TopBP1 coprecipitated with total treslin. As with S1000 phosphorylation,

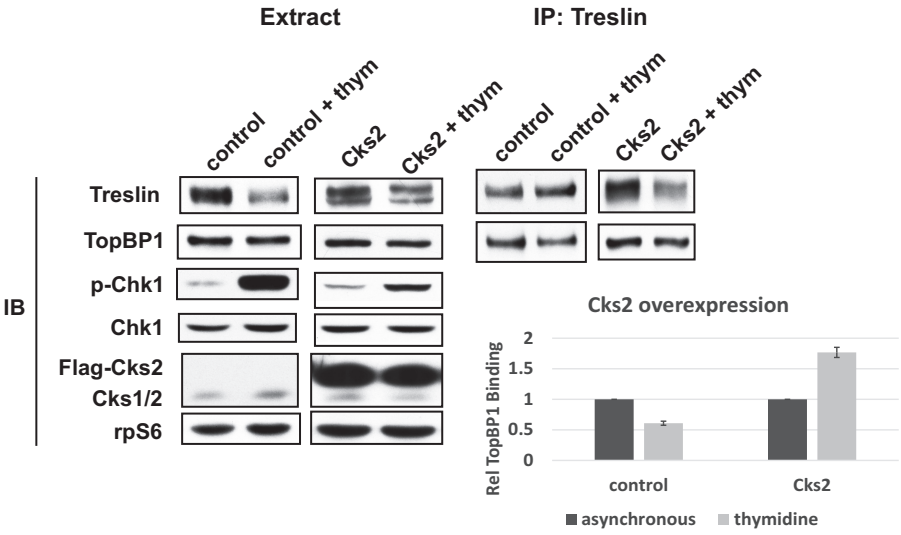

FIG 8 Overexpression of Cks2 prevents dephosphorylation of treslin during replication stress. Control HeLa cells or HeLa cells stably overexpressing Flag-Cks2 were treated with 2 mM thymidine (thym) for 1 h to trigger the replication stress checkpoint. Extracts were prepared from untreated and thymidine-treated cells and immunoprecipitated with antitreslin antibody. Immunoprecipitates were analyzes by SDS-PAGE and immunoblotting (IB) using anti-TopBP1 and treslin antibodies, as described in the legend to Fig. 7. The bar graph represents quantification of data from 2 independent biological experiments. Error bars correspond to SD. (Left) Extracts were analyzed for the indicated proteins. p-Chk1 indicates Chk1 phosphorylated on serine 345, a target of ATR kinase confirming replication stress. Ribosomal protein S6 (rpS6) represents the loading control.

silencing of Cks1 and Cks2 produced an ~70% reduction in TopBP1 binding to treslin ($P = 0.005$ by single-tailed *t* test) (Fig. 7C). Based on levels of cyclins E and A in the lysates (Fig. 7C), which were comparable between control and Cks1/2-silenced samples, it is unlikely that differences in treslin S1000 phosphorylation can be attributed to a reduction in Cdk2 kinase activity resulting from CKS silencing. In addition, after a 24-h interval of silencing of Cks1 and Cks2, there is no significant change in the cell cycle distribution of the population relative to that of asynchronous controls (Fig. 7B).

CKS protein overexpression prevents dephosphorylation of treslin in response to the replication stress checkpoint. CKS protein overexpression overrides the replication stress checkpoint. We hypothesized that this is due to an inability to dephosphorylate treslin in the presence of high levels of either Cks1 or Cks2. To test this possibility, we compared control HeLa cells to HeLa cells overexpressing Cks2. We employed Cks2 in these experiments because, unlike Cks1, Cks2 is not involved in the SCF-dependent turnover of CDK inhibitors. Thus, any results attributable to Cks2 overexpression are unlikely to be a result of changes in the levels of CDK inhibitors. The replication stress checkpoint was triggered, as indicated by phosphorylation of Chk1 (Fig. 8), by incubating cells in 2 mM thymidine for 1 h. Treslin immunoprecipitates were compared for levels of coprecipitated TopBP1 as a surrogate for treslin S1000 phosphorylation, as validated in Fig. 7. We felt that this was a more accurate way to measure relative treslin phosphorylation, since TopBP1 and treslin ratios were determined from the same immunoprecipitate. For control HeLa cells, thymidine treatment led to an almost 50% decrease in TopBP1 binding, consistent with dephosphorylation of treslin in response to the replication stress checkpoint. However, for HeLa cells overexpressing Cks2, no decrease in TopBP1 binding in response to thymidine treatment was observed ($P = 0.0015$ comparing thymidine-treated samples using single-tailed *t* test) (Fig. 8), consistent with override of the replication stress checkpoint in these cells. Indeed, Cks2 overexpression leads to an increase in TopBP1 binding upon replication stress, which correlates with an increase in origin activation observed under these conditions (34). If CDK binding is not required to enhance treslin phosphorylation, then overexpression of a mutant CKS protein deficient in CDK binding should similarly override the replication

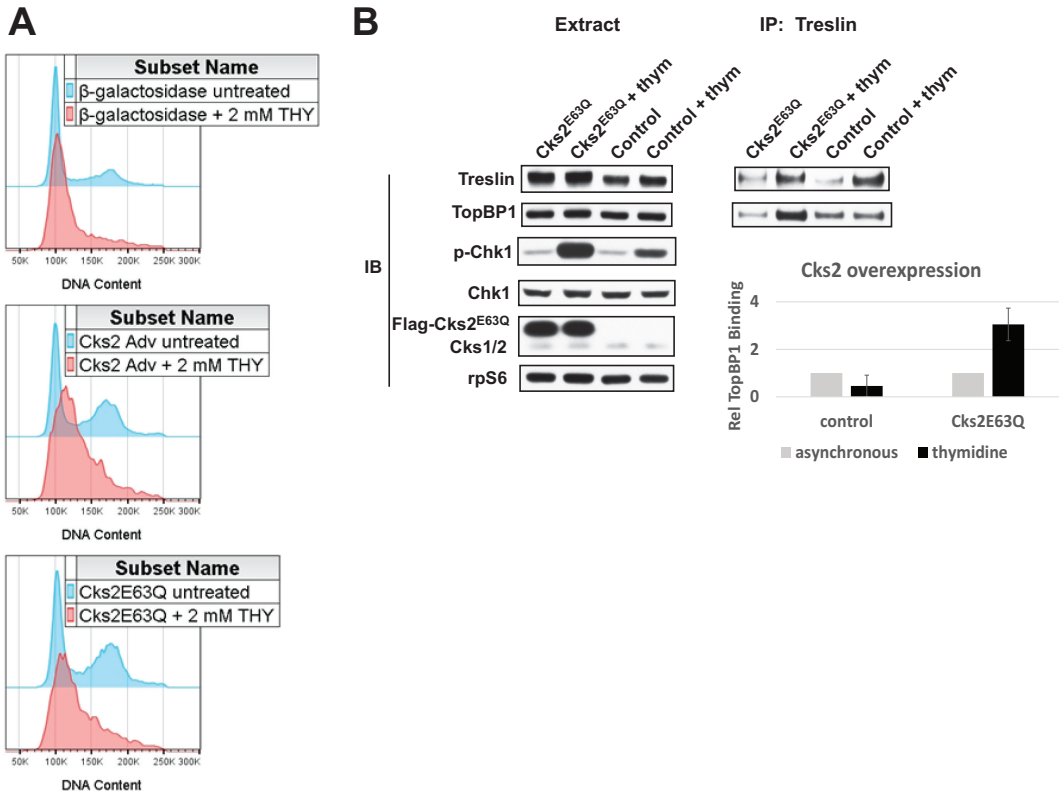

FIG 9 Overexpression of CDK binding-defective mutant Cks2E63Q prevents dephosphorylation of treslin during replication stress. Control HeLa cells or HeLa cells transiently overexpressing Flag-Cks2 or Flag-Cks2 E63Q by adenoviral transduction were treated with 2 mM thymidine for 1 h to trigger the replication stress checkpoint. Cultures were analyzed for cell cycle distribution by FACS (A), or extracts were prepared from untreated and thymidine-treated cells and immunoprecipitated with antitreslin antibody (B). "Cks2 Adv" refers to cells transduced with a recombinant adenovirus programmed to overexpress Cks2. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using anti-TopBP1 and treslin antibodies, as described in the legend to Fig. 8. The bar graph represents quantification of data from 2 independent biological experiments. Error bars correspond to SD. (Left) Extracts were analyzed for the indicated proteins. p-Chk1 indicates Chk1 phosphorylated on serine 345, a target of ATR kinase confirming replication stress. Ribosomal protein S6 (rpS6) represents the loading control.

stress checkpoint by maintaining treslin phosphorylation. Indeed, overexpression of Cks2 E63Q overrides the replication checkpoint (Fig. 9A) and maintains treslin phosphorylation, as indicated by TopBP1 binding (Fig. 9B), similar to wild-type Cks2 ($P = 0.02$ for comparisons of thymidine-treated samples using single-tailed t test), confirming that canonical Cdk2 binding is not required for stimulation of treslin phosphorylation in cells.

Cks1/2 induction correlates with and is required for checkpoint recovery. We hypothesized that Cks1/2-mediated override of the replication stress checkpoint is relevant to checkpoint adaptation or recovery. Consistent with this, we observed that Cks1/2 mRNA and protein levels increased rapidly upon removal of thymidine (2 h) even before observable progression through S phase had occurred (Fig. 10A and B). More significantly, silencing of Cks1 and Cks2 rendered cells incapable of recovering from the replication stress checkpoint (Fig. 11A). On the other hand, overexpression of Cks2 promoted more rapid recovery from the checkpoint, as cells could be observed progressing through S phase even at the 2-h time point (Fig. 11B).

DISCUSSION

CKS proteins as cofactors for treslin phosphorylation. Roles have been described previously for CKS proteins as adaptors that link CDKs to substrates (6, 7). In particular, the ability of CKS proteins to bind phosphates suggests a mechanism whereby a CKS protein, by tethering a CDK to an already phosphorylated protein, can promote the processive phosphorylation of additional nearby sites. Although the current study does

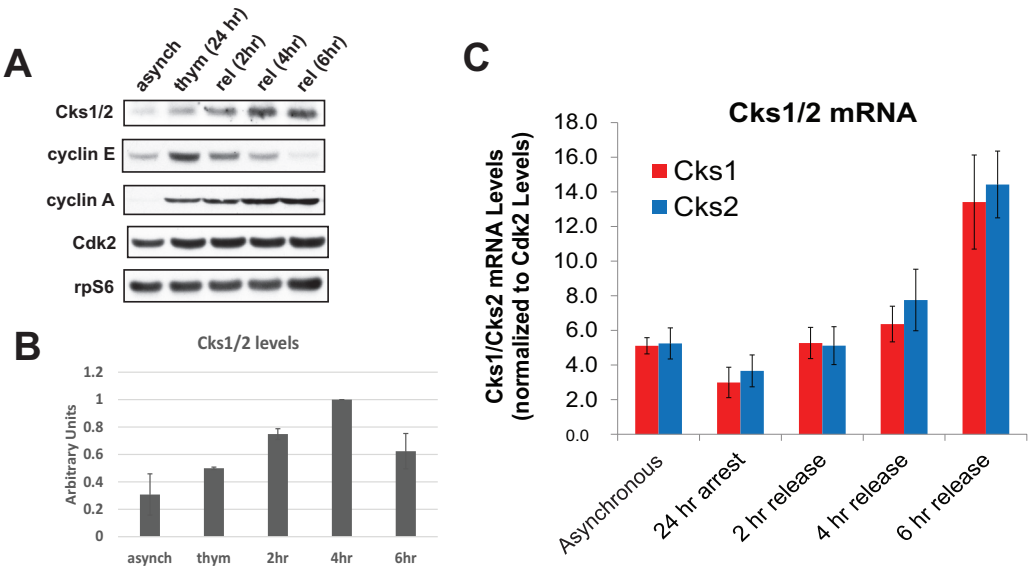

FIG 10 Induction of Cks1 and Cks2 promotes recovery from the replication stress checkpoint. Cks1 and Cks2 levels rise immediately upon reversal of replication stress. HeLa cells were treated with 2 mM thymidine for 24 h, after which thymidine was washed out and cells were incubated with fresh medium without thymidine. (A) Extracts were prepared from asynchronous (asynch) cells, thymidine-arrested cells, and at 2, 4, and 6 h after release from the thymidine block. SDS-PAGE and immunoblotting were carried out to analyze the indicated proteins. (B) The bar graph represents quantification of the combined levels of Cks1 and Cks2 relative to ribosomal protein S6 from two independent biological experiments. Image data from the two experiments were normalized such that the signal at the time point corresponding to 4 h after release was set to 1.0. Error bars correspond to SD. (C) Analysis of CKS1 and CKS2 mRNA by quantitative real-time PCR after a thymidine block and release. Cells were incubated in 2 mM thymidine for 24 h and then released. Cells were harvested at the indicated times and mRNA was prepared for qRT-PCR. Signals for Cks1 and Cks2 were normalized to Cdk2 mRNA. Data correspond to three independent biological experiments. Error bars represent SD.

not preclude such a mechanism for treslin, which contains numerous potential CDK phosphorylation sites, it suggests an additional novel mechanism for treslin S1000 phosphorylation, the key site for origin firing. Based on experiments with a treslin-derived peptide containing the S1000 site, non-CDK-bound CKS protein can promote CDK-dependent phosphorylation. However, the data presented are not consistent with a simple model where CKS protein binds to treslin prior to engagement by the kinase. Specifically, the weak binding of CKS protein to treslin in the absence of Cdk2 is inconsistent with the EC_{50} of CKS protein-dependent stimulation of treslin phosphorylation. We therefore propose two potential models. In the first, treslin first engages Cdk2 to form a low-affinity complex that is inefficient for phosphorylation (Fig. 12A). In this complex, however, treslin is remodeled to form the actual target of CKS protein binding, converting it to a high-affinity complex that is efficient for phosphorylation (Fig. 12A). In the second model, CKS proteins serve as noncanonical adapters by binding to both Cdk2 and treslin in a manner not previously described (5) and requiring different contact residues (Fig. 12B). This mechanism would be similar to the adapter function for Cks1 observed for binding of the ubiquitin ligase substrate-binding subunit Skp2 to the CDK inhibitor $p27^{Kip1}$ (11, 12). Both of these models are consistent with a number of observations. (i) CKS protein mutants shown to be incapable of CDK binding can still stimulate S1000 phosphorylation, indicating that the previously described canonical "adapter" function can be excluded. (ii) CKS proteins are capable of stimulating phosphorylation of an 8-residue peptide containing the S1000 site. An interaction between CKS protein and Cdk2, as structurally determined, is not compatible with a simultaneous interaction between CKS protein and the substrate peptide bound to the active site of Cdk2 (5). (iii) However, CKS proteins bind only weakly to free treslin peptide. (iv) On the other hand, CKS proteins alter the K_m for treslin phosphorylation. We therefore speculate that the role of CKS protein in this context is to produce a high-affinity complex conducive to efficient phosphorylation by Cdk2, although the

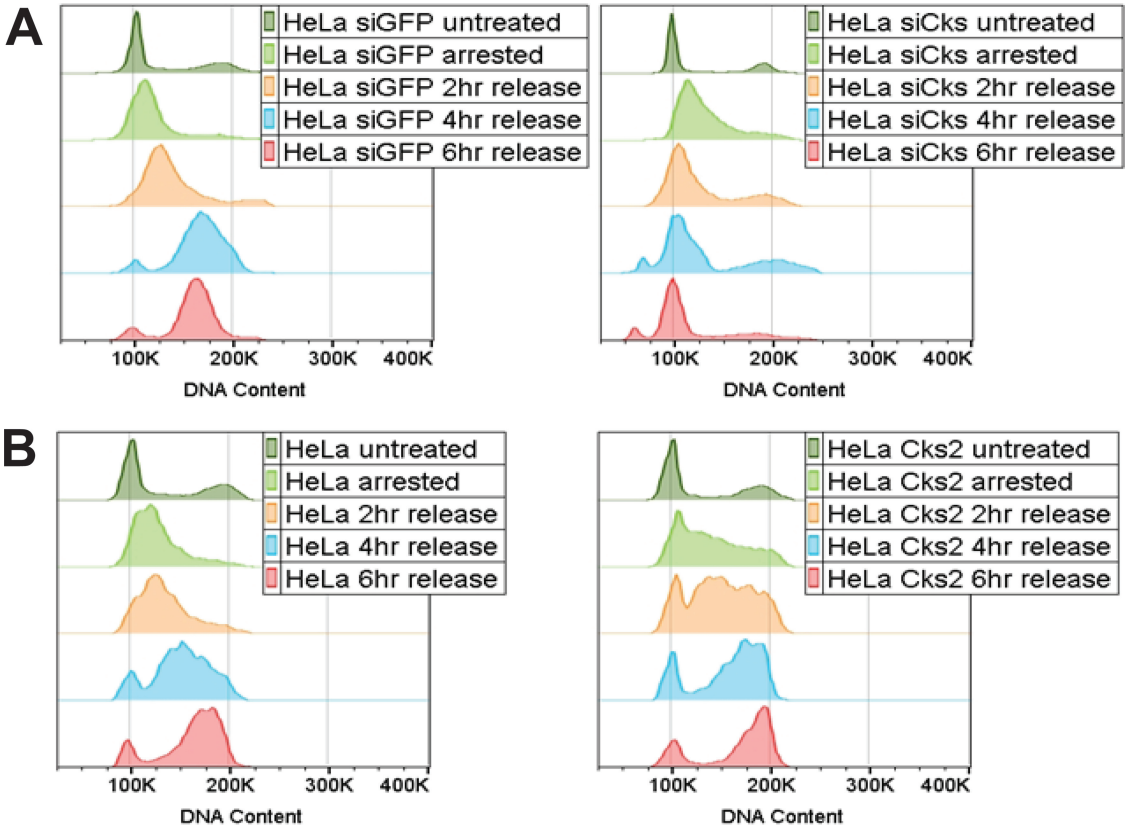

FIG 11 Flow-cytometric analysis of control HeLa cells, HeLa cells silenced for Cks1 and Cks2 (A), and HeLa cells stably overexpressing Cks2 treated with 2 mM thymidine for 24 h and then released for the indicated times from the thymidine block (B). The abscissa represents DNA content and the ordinate represents the number of cells.

data at this point cannot distinguish between the CKS protein binding site consisting of treslin residues or a combination of treslin and Cdk2 residues (Fig. 12).

CKS proteins and override of the replication stress checkpoint. Cells overexpressing Cks1 or Cks2 fail to stop origin firing when the replication stress checkpoint is triggered, leading to continued progression through S phase (34). Nevertheless, the replication stress signaling pathway was shown to be intact, with no defect in the inhibitory phosphorylation of Cdk2 on Y15. However, based on our studies, treslin continues to be phosphorylated, presumably by Cdk2. Two possible mechanisms could

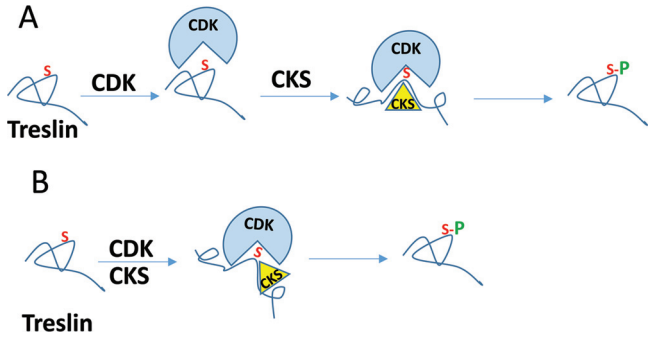

FIG 12 Potential mechanisms of CKS protein-dependent stimulation of treslin phosphorylation. (A) Cdk2 forms a low-affinity, inefficient complex with treslin. However, this binary complex forms a binding site for CKS proteins on treslin that converts it to a high-affinity complex that is efficient for phosphorylation. (B) CKS proteins are noncanonical adapters that bind both Cdk2 and treslin to produce a high-affinity complex efficient for phosphorylation. This would be similar to the adapter role that Cks1 plays in linking Skp2 to $p27^{Kip1}$.

account for this observation. CKS protein could serve to improve treslin as a substrate for Y15-phosphorylated Cdk2. Alternatively, the ability of residual nonphosphorylated Cdk2 to phosphorylate treslin could be enhanced by CKS protein binding. We favor the latter explanation, as the crystallographically determined structure of Y15-phosphorylated Cdk2 is not compatible with the ability to accommodate a protein substrate in the active-site groove (47). Indeed, it is evident that even when checkpoint signaling is active, the steady state of Y15 phosphorylation of Cdk2 is considerably less than 100%, as Cdk2 kinase activity is easily detectable (34, 48). If CKS protein saturation can increase the efficiency of S1000 phosphorylation 10- to 20-fold, then only a small fraction of Cdk2 would need to be active to maintain treslin in a phosphorylated state.

The role of CKS-dependent checkpoint override. Cks1 and/or Cks2 are frequently overexpressed in a broad spectrum of human malignancies, and we have shown that the replication stress checkpoint cannot block DNA replication in cell lines derived from such cancers (34). Therefore, we have speculated that CKS protein overexpression is selected to evade oncoprotein-induced replication stress. However, this cannot be the evolutionarily selected role for CKS protein-dependent checkpoint override. In the current study, we show that Cks1 and Cks2 expression is significantly increased immediately upon reversal of replication stress and that silencing of Cks1 and Cks2 prevents recovery from the checkpoint and resumption of DNA replication, suggesting that CKS protein-mediated checkpoint override has a role in facilitating recovery.

MATERIALS AND METHODS

Cell lines and cell culture. HeLa cells were programmed to overexpress Cks1 or Cks2 by transduction with recombinant retroviruses (pBABE-puro-Cks1 or Cks2) (34) or, in the case of Cks2 E63Q, recombinant adenoviruses (34). In the case of Cks2 E63Q, adenoviral transduction was necessary to achieve overexpression similar to retrovirally mediated expression of Cks2. Retroviral controls were cells transduced with empty vector. All experiments were carried out with mixed populations of transduced cells. Cells were plated onto 15-cm plates (Corning Inc.) at 5×10^6 cells/plate for 24 h in Dulbecco's modified Eagle's medium (DMEM). For silencing experiments, cells were treated with short interfering RNA (siRNA) oligonucleotides targeting green fluorescent protein (GFP), Cks1, Cks2, or Cks1 and Cks2 for 24 h before harvesting. For replication stress experiments, cells were treated with 2 mM thymidine for 1 h before harvesting. For experiments where cells were transduced with recombinant adenovirus, transductions were carried out 24 h prior to harvest. Once pelleted, cells were lysed in NP-40 lysis buffer (49) with 2× the manufacturer's recommended concentration of protease and phosphatase inhibitors (Roche cOmplete protease inhibitor cocktail and PhoSTOP phosphatase inhibitor cocktail). Protein concentration was determined via the Bradford protein assay (Bio-Rad).

Fluorescence-activated cell sorter (FACS) analysis. Cells were plated in 10-cm plates (Corning Inc.) at 6×10^5 per plate for 24 h. Once harvested, cells were fixed overnight in a 70:30 ethanol-phosphate-buffered saline (PBS) solution. After overnight fixation, cells were pelleted and permeabilized in 1 ml of 1% bovine serum albumin (BSA)–0.5% Tween 20–PBS. RNase solution was made by boiling 10 mg of RNase in 1 ml of 15 mM NaCl–50 mM Tris solution for 15 min. One milliliter of RNase solution was combined with 9 ml of PBS–0.1% Triton X-100 and 200 μl of 10 mg/ml propidium iodide. After the cells were pelleted by centrifugation, pellets were resuspended in propidium iodide staining solution and incubated at room temperature for 2 h before flow-cytometric analysis (NovoCyte; ACEA Biosciences). Data analysis was carried out using FlowJo v.10.1.

Recombinant proteins. Purification of CKS proteins and GST-treslin peptide fusions has been described previously (40, 50). Active 6×His-cyclin E-Cdk2 was purified from an *E. coli* strain expressing yeast Cak1 (43) by nickel chelate chromatography followed by gel filtration chromatography.

Quantitative real time-PCR analysis (qRT-PCR). Control and Cks1/2-overexpressing HeLa cells were plated on 15-cm plates (Corning Inc.) at 5×10^6 cells for 24 h. Cells were transfected with siRNA oligonucleotides targeting GFP, Cks1, Cks2, or Cks1 and Cks2 (Qiagen) at a concentration of 10 nM for 24 h using Lipofectamine RNAiMax (Life Technologies). RNAs were harvested using RNeasy minikit columns (Qiagen). RNA (20 ng/μl) was reversed transcribed, amplified, and quantified using PerfeCTa Sybr green Supermix (Quanta BioSciences). Each sample was run in octuplicates. Expression levels were determined relative to the control mRNA *CDK2* using the formula $2^{CT(\text{control mRNA}) - CT(\text{mRNA of interest})} \times 10{,}000$, where C_T is the threshold cycle. Standard deviations (SD) of the relative expression values were calculated. Primers were obtained from Integrated DNA Technology. Primers were the following: *CDK2*, 5′ GTTGAGGAACTGAGATGCGG 3′ (forward) and 5′ TTGTTCTTGGATGTGGGGAG 3′ (reverse); *CKS1*, 5′ CTGATGTCTGAATCTGAATGGAGG 3′ (forward) and 5′ TTTCTTTGGTTTCTTGGGTAGTGG 3′ (reverse); *CKS2*, GAAGAGGAGTGGAGGAGACTT (forward) and 5′ GTTGATCTTTTGGAAGAGGTCGT 3′ (reverse).

***In vitro* kinase assays.** Reaction mixtures of 30 μl consisted of 17 nM activated Cdk2/CycE purified from *E. coli*, 1.1 μM GST-treslin, and the indicated concentrations of CKS protein and were incubated in kinase buffer (20 mM Tris-HCl [pH 7.5], 2.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 250 μM ATP) at 37°C for 1 h. Reactions were quenched by addition of SDS-PAGE sample buffer and heating.

Thermal shift assays. Thermal shift assays in 20 μl of kinase buffer contained 10 μM Cks1 alone or Cks1 and treslin (997-1008) peptide at concentrations ranging from 10 μM to 25 μM. SyproOrange dye was diluted 8× from the stock provided by the manufacturer (Applied Biosystems) in the protein thermal shift dye kit. Reactions were carried out in octuplicate in a 96-well plate. Reaction mixtures were first incubated at 4°C for 1 h, and then a Bio-Rad CFX Connect qRT-PCR thermocycler was programmed for 0.5°C steps from 25°C to 95°C. After each step the fluorescence emission was read to generate a melt curve. The maximum value of the derivative of the melt curve (dRFU/dT) was taken as the melting point (T_m).

Determination of K_ms for treslin phosphorylation. To determine the K_m for treslin phosphorylation, GST-treslin (989-1011) at increasing concentrations was incubated with cyclin E-Cdk2 at 17 nM under conditions described above for kinase reactions. Reaction mixtures contained either no CKS protein or 25 μM Cks1 or Cks2. Reactions were analyzed by SDS-PAGE and Western blotting. However, a number of modifications were made to improve quantification. Gels were loaded such that equal amounts of GST-treslin were in each lane so that transfer artifacts, such as membrane saturation, were avoided. Signals were then deconvolved to give a final adjusted value. After incubation of membranes with antiphosphotreslin (S1000) antibody, quantification was carried out using a Li-Cor Odyssey infrared imager set so that all readings were in the linear range, rather than using film, which generally is not linear. Data were plotted and analyzed using GraphPad Prism curve-fitting software. Each experiment was composed of duplicate samples at each treslin concentration, and each experiment was carried out twice.

Mass spectrometry. The peptides were infused directly into an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) using electrospray ionization (ESI) at about 500 nl/min. To record *m/z* values for intact peptides, the instrument was scanned at a resolution of 60,000 for about 10 min. Tandem MS data were collected for *m/z* values at 770.3 and 810.3, representing the unmodified and modified peptide, respectively.

Antibodies. Primary antibodies were obtained from the following sources: Cks1/2 (FL-79; Santa Cruz Biotechnology), treslin and serine 1000-phosphorylated treslin (40, 46), Chk1 (26105; Cell Signaling Technology), serine 345-phosphorylated Chk1 (13303; Cell Signaling Technology), cyclin A (H432; Santa Cruz Biotechnology), cyclin E1 (E12; Santa Cruz Biotechnology), Cdk2 (M2; Santa Cruz Biotechnology), TopBP1 (A300-111A; Bethyl Laboratories), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (3781; ProSci), and ribosomal protein S6 (5610; Cell Signaling Technology). For Western blotting, all antibodies were used at 1:1,000. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies were from Jackson Laboratories and used at 1:2,500. For immunoprecipitation experiments, 1.5 μg of either treslin or phospho-S1000 treslin antibody was used per sample, and HRP-conjugated protein A (Clean-Blot; Thermo Scientific) was used instead of a second antibody.

Immunoprecipitation, electrophoresis, and blotting. Once adjusted to equal concentrations, cell lysates were immunoprecipitated in the presence of 50 μg/ml ethidium bromide for 3 h with anti-human treslin or antitreslin pS1000 antibody. Immune complexes were recovered by incubation with protein A Dynabeads (Life Technologies) for 1 h. After incubation, beads were washed three times with NP-40 buffer containing 2× the manufacturer's recommended concentration of protease and phosphatase inhibitors (Roche cOmplete protease inhibitor cocktail and PhoSTOP phosphatase inhibitor cocktail), and proteins then were eluted by incubation in SDS-PAGE sample buffer for 10 min at 65°C. For immunoblotting, lysates were separated on precast gradient SDS-PAGE gels (Life Technologies) and transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane using an iBlot gel transfer device (Life Technologies). After blocking in 5% (wt/vol) milk made in 1× TBS-Tween 20 for 1 h at room temperature, the membranes were incubated with primary antibody overnight at 4°C, washed, and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Blots were developed using ECL technology. Quantification of films was carried out using ImageJ.

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S.I.R. supervised the research described. R.M., J.T., Y.Z., and S.I.R. designed and carried out experiments. R.Z. carried out experiments. A.K. and W.G.D. provided advice and reagents. S.I.R. wrote the manuscript, with editing help from A.K., W.G.D., J.T., and J.R.Y. J.R.Y. provided experimental resources.

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